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16. ABSTRACT

Two groups of compounds were selected for microbial transformation studies. In the first group were carboxylic acid esters having a fixed aromatic moiety and an increasing length of the alkyl component. Ethyl esters of chlorine-substituted carboxylic acids were in the second group. Microorganisms from environmental waters and a pure culture of Pseudomonas putida U were used. The bacterial populations were monitored by plate counts, and disappearance of the parent compound was followed by gas-liquid chromatography as a function of time. The products of microbial hydrolysis were the respective carboxylic acids. Octanol-water partition coefficients (K_{ow}) for the compounds were measured. These values spanned three orders of magnitude, whereas microbial transformation rate constants (k_b) varied only 50-fold. The microbial rate constants of the carboxylic acid esters with a fixed aromatic moiety increased with an increasing length of alkyl substituents. The regression coefficient for the linear relationships between log k_b and log K_{ow} was high for group 1 compounds, indicating that these parameters correlated well. The regression coefficient for the linear relationships for group 2 compounds, however, was low, indicating that these parameters correlated poorly.

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Microbial Transformation of Esters of Chlorinated Carboxylic Acids

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Two groups of compounds were selected for microbial transformation studies. In the first group were carboxylic acid esters having a fixed aromatic moiety and an increasing length of the alkyl component. Ethyl esters of chlorine-substituted carboxylic acids were in the second group. Microorganisms from environmental waters and a pure culture of *Pseudomonas putida* U were used. The bacterial populations were monitored by plate counts, and disappearance of the parent compound was followed by gas-liquid chromatography as a function of time. The products of microbial hydrolysis were the respective carboxylic acids. Octanol-water partition coefficients (K_{ow}) for the compounds were measured. These values spanned three orders of magnitude, whereas microbial transformation rate constants (k_b) varied only 50-fold. The microbial rate constants of the carboxylic acid esters with a fixed aromatic moiety increased with an increasing length of alkyl substituents. The regression coefficient for the linear relationships between log k_b and log K_{ow} was high for group 1 compounds, indicating that these parameters correlated well. The regression coefficient for the linear relationships for group 2 compounds, however, was low, indicating that these parameters correlated poorly.

Structure-activity relationships have been used for many years in developing and designing drugs and pesticides. In pesticide design, for example, they are used to evaluate the most effective substituents of a molecule on the target organism, such as the effects of chain branching in phenoxyacetic acid esters on weeds (3). Our interest, however, resides in defining the effect of substituents on the kinetics of microbial transformation of chemical compounds. Quantitative relationships between microbial rate constants and the physical or chemical properties of a group of congeners have been reported for selected pesticides (18), phthalates (18), and phenols (10, 11). Using these relationships, environmentalists have a powerful tool to assess the fate and effects of existing and new organic compounds in aquatic systems.

To illustrate the relation between microbial transformation and hydrophobicity of a molecule, we selected two groups of compounds for these studies. In the first group were carboxylic acid esters of a fixed aromatic moiety (2,4-dichlorophenoxyacetate [2,4-D]) with an increasing length of the alkyl component. In the second group were ethyl esters of chlorine-substituted phenoxyacetic acids. This report presents kinetic data on the microbial transformation for a series of these compounds. Correlations of rate constants with hydrophobicity are also illustrated.

MATERIALS AND METHODS

Organism. Stock cultures of *Pseudomonas putida* U (courtesy of Peter Chapman, University of Minnesota) were maintained as slant cultures on tryptone glucose extract agar (Difco Laboratories, Detroit, Mich.).

Water collection. Water samples were collected from four ponds and one river within 10 km of Athens, Ga., and transported to the laboratory in sterile glass jugs. Kinetic studies generally were initiated within 5 h after sample collection; sequential studies were made within 96 h.

Materials. Esters of 4-chlorophenoxyacetic acid, 2,4-D, and 2,4,5-trichlorophenoxyacetic acid were prepared by acid-catalyzed reaction of the commercial acids (Aldrich Chemical Co., Milwaukee, Wis.) with the appropriate alcohols. Esters were purified by fractional distillation.

Ethyl esters of 2,3,4,5-tetrachlorophenoxyacetic acid and pentachlorophenoxyacetic acid were prepared by the following procedure, suggested by Albert Leeper, Chalfont, Pa. (personal communication). Pentachlorophenol (Eastman Chemical Products, Inc., Rochester, N.Y.) or 2,3,4,5-tetrachlorophenol (Aldrich) and sodium hydroxide were added to 100 ml of absolute ethyl alcohol in a reaction vessel which was stirred and refluxed under nitrogen in an oil bath. As the mixture boiled, ethyl bromoacetate (Eastman) and sodium-dried xylene were added and the alcohol was distilled. When the reaction was complete, the mixture was cooled and washed with water to remove unreacted sodium penta- or tetrachlorophenoate. The organic phase (xylene) was filtered, and the residue was dissolved in hot ethyl alcohol-water (3:1 for pentachlorophenoate and 2:1 for tetrachlorophenoate). The solution was cooled, and the compound was crystallized.

2,3,4,5-Tetrachlorophenoxyacetic acid (mp 158°C) and pentachlorophenoxyacetic acid (mp 175 to 176°C), used as thin-layer chromatography standards, were prepared by alkaline hydrolysis of the ethyl esters. Structures of the acids and esters were confirmed by nuclear magnetic resonance and direct-probe mass spectrometry. No impurities were detected by gas chromatography with an electron-capture detector.

Gas-liquid chromatography. Aqueous samples of the reaction mixtures were extracted with acetonitrile followed by isooctane. Samples were mixed vigorously in a vortex mixer for 1 min. Isooctane extracts of the reaction mixtures were analyzed on a gas chromatograph with a ^{63}Ni electron-capture detector and a column (1 m long) packed with 3% SE-30 on Gas-Chrom Q (Supelco, Inc., Bellefonte, Pa.). Oven temperatures were 180°C for the ethyl ester of 4-chlorophenoxyacetic acid; 190°C for the methyl and ethyl esters of 2,4-D; 210°C for the propyl ester of 2,4-D and the ethyl esters of 2,4,5-trichlorophenoxyacetic acid, 2,3,4,5-tetrachlorophenoxyacetic acid, and pentachlorophenoxyacetic acid; and 225°C for the butyl, hexyl, and octyl esters of 2,4-D.

Thin-layer chromatography. Reaction mixtures were adjusted to pH 2 with 2 M hydrochloric acid and extracted

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twice with methylene chloride. Products in the extracts were separated by thin-layer chromatography, using plates coated with fluorescent silica gel. The developing solvent system was benzene-acetic acid (8:2). Visualization was accomplished with a chromatographic viewer with a UV (short-wave) lamp.

Octanol-water partition coefficients and water solubilities. Octanol-water partition coefficients (K_{ow}) were calculated by the procedures of Karickhoff and Brown (5). Analyses of the two phases were carried out by gas-liquid chromatography of the isooctane extract of the aqueous layer and the octanol layer after dilution with hexane.

Water solubilities of the esters were determined under sterile conditions by using a specially constructed vessel described previously by Perdue and Wolfe (13). Aqueous samples were extracted with isooctane and analyzed by gas-liquid chromatography.

Design of degradation experiments. For pure culture studies, *P. putida* was cultured in 1-liter Erlenmeyer flasks containing 500 ml of dilute (1:10) nutrient broth that had been autoclaved at 121°C for 15 min. After 24 h, bacteria were harvested, washed three times with sterile dilution water, and suspended in 1 liter of basal salts medium (pH 6.7) as described previously by Payne and Feisal (12). About 200 ml of the suspension was transferred to each of five 500-ml flasks. Sterile basal salts medium served as a control for abiotic processes.

Approximately 200 ml of each environmental water sample was transferred to each of five 500-ml flasks. Filter-sterilized environmental water samples served as controls for abiotic processes.

A 10- to 20-μl acetone solution of each compound was used to introduce the test compound into four nonsterile flasks and one sterile flask. Flask 6 served as a control to detect changes in bacterial population not due to the presence of the xenobiotic. Flasks were incubated at 25°C in a temperature-controlled shaker. Bacterial concentrations in the selected water samples ranged from 5×10^{7} to 1×10^{9} organisms per liter. Concentrations of 0.01 and 0.001 mg/liter were used for all of the compounds except the ethyl ester of 4-chlorophenoxyacetic acid, for which concentrations of 0.1 and 0.01 mg/liter were used. All experiments were short term (not longer than 48 h), and the bacterial concentrations remained constant throughout the study.

Bacterial enumerations and treatment of the transformation kinetics data described previously (9) were used.

RESULTS

Our studies indicate that the esters were readily transformed by the five environmental populations and *P. putida* (Tables 1 and 2), suggesting a wide occurrence of nonspecific esterases in microorganisms as has been previously reported (18). Other researchers (1) also have reported phenoxyacetic acid esters to be easily transformed by environmental populations. At 24-h sampling periods, no more than 50% of all of the compounds remained. Microbial rate constants for the 2,4-D esters rangee from 5.9×10^{-10} liters · organism^{-1} · h^{-1} for the methyl ester to 3.5×10^{-8} liters · organism^{-1} · h^{-1} for the octyl ester, about a 50-fold difference. For the eight determinations per site, four high and four low concentrations of the xenobiotics were used for the aqueous samples. The highest relative standard deviation was 82% for the 2,4-D ethyl ester and site 5. This particular site (a pond) showed greater differences among the rate constant values for samples collected at different times than did the other sites. A similar observation was made for this site in the studies with butyl and octyl esters of 2,4-D.

In contrast to the 2,4-D ester studies, the group of ethyl esters and environmental populations showed little difference between the transformation rate constants of 4-chlorophenoxyacetic acid ester and pentachlorophenoxyacetic acid ester (Table 2). This small difference between the rate constants for transformation of the most lipophilic and least lipophilic compounds of group 2 resulted in using pure cultures so that slight differences among the rate constants for the five compounds could be better defined. The transformation rate constants in the pure culture studies were an order of magnitude lower than in the environmental waters. This phenomenon has been observed previously in our laboratory and other laboratories (2, 11). The microbial transformation rate constant (k_b) values among the compounds in Table 2 for *P. putida*, however, showed only a threefold difference.

Thin-layer chromatographic analyses of the organic extracts of the samples indicated that the chlorinated carboxylic acids of the esters studied were the major products. Samples were cochromatographed with authentic standards of the acids. Rate constants determined in these studies were for microbial hydrolysis of the parent compounds.

The results of the water solubility experiments are summarized in Table 3. Two reaction vessels with different quantities of the compound were used to determine solubilities. At 25°C solubilities of the 2,4-D *n*-alkyl esters had a range of four orders of magnitude, and the ethyl esters with chlorine substituents on the benzene ring had a range of almost five orders of magnitude. The solubility of the 2,4-D octyl ester (0.101 ± 0.01 μM) was in close agreement with the value of 0.101 ± 0.014 μM reported previously by Perdue and Wolfe (13). The methyl, butyl, and octyl ester solubilities in Table 3 agreed within a factor of two with the values reported by Zepp et al. (20).

DISCUSSION

The magnitude of the transformation rate constants of the 2,4-D esters increased as the alkyl length increased. Few

TABLE 1. k_b values for six esters of 2,4-D and five sites

2,4-D ester	k_b (mean liters · organism^{-1} · h^{-1} ± SE)[a] at site no.:					
	1	2	3	4	5	Mean for all sites
Methyl	$5.9 (\pm 2.4) \times 10^{-10}$	$7.3 (\pm 2.0) \times 10^{-10}$	$5.3 (\pm 3.0) \times 10^{-10}$	$5.2 (\pm 1.6) \times 10^{-10}$	$5.5 (\pm 2.0) \times 10^{-10}$	$5.8 (\pm 0.9) \times 10^{-10}$
Ethyl	$7.5 (\pm 2.3) \times 10^{-10}$	$5.4 (\pm 1.9) \times 10^{-10}$	$4.2 (\pm 1.1) \times 10^{-10}$	$5.5 (\pm 1.6) \times 10^{-10}$	$3.3 (\pm 2.7) \times 10^{-10}$	$5.2 (\pm 1.6) \times 10^{-10}$
Propyl	$3.1 (\pm 2.2) \times 10^{-9}$	$3.7 (\pm 2.7) \times 10^{-9}$	$1.7 (\pm 1.1) \times 10^{-9}$	$1.6 (\pm 0.3) \times 10^{-9}$	$4.2 (\pm 2.1) \times 10^{-9}$	$2.9 (\pm 1.2) \times 10^{-9}$
Butyl	$4.1 (\pm 2.7) \times 10^{-9}$	$4.5 (\pm 1.8) \times 10^{-9}$	$4.6 (\pm 1.4) \times 10^{-9}$	$4.1 (\pm 2.1) \times 10^{-9}$	$3.1 (\pm 2.2) \times 10^{-9}$	$4.1 (\pm 1.2) \times 10^{-9}$
Hexyl	$3.6 (\pm 1.7) \times 10^{-8}$	$2.2 (\pm 1.0) \times 10^{-8}$	$1.0 (\pm 0.1) \times 10^{-8}$	$2.2 (\pm 0.7) \times 10^{-8}$	$1.9 (\pm 0.4) \times 10^{-8}$	$4.2 (\pm 3.3) \times 10^{-8}$
Octyl	$1.6 (\pm 0.7) \times 10^{-8}$	$4.0 (\pm 3.0) \times 10^{-8}$	$3.6 (\pm 2.0) \times 10^{-8}$	$2.5 (\pm 0.9) \times 10^{-8}$	$4.2 (\pm 3.3) \times 10^{-8}$	$3.2 (\pm 1.1) \times 10^{-8}$

[a] Values represent the mean of eight determinations per site with the standard error of the estimate.

TABLE 2. k_b values for transformations of esters of chlorinated phenoxyacetic acids

Ethyl ester of:	k_b (mean liters · organism^{-1} · h^{-1} ± SD)[a]			
	P. putida ($\times 10^{-11}$)	Site 1 ($\times 10^{-10}$)	Site 3 ($\times 10^{-10}$)	Site 5 ($\times 10^{-10}$)
4-Chlorophenoxyacetic acid	2.3 ± 0.9	2.5 ± 1.3	4.4 ± 2.1	3.4 ± 1.2
2,4-D	5.0 ± 1.2			
2,4,5-Trichlorophenoxyacetic acid	4.7 ± 2.1			
2,3,4,5-Tetrachlorophenoxyacetic acid	6.3 ± 0.9			
Pentachlorophenoxyacetic acid	4.5 ± 1.2	1.9 ± 0.6	4.1 ± 0.5	2.8 ± 0.3

[a] Values represent the means and standard deviations of 12 determinations for *P. putida* and 8 determinations for sites 1, 3, and 5.

studies (16, 19) relating the hydrophobic properties of a series of congeners to their microbial transformation rate constants have been reported. Yonezawa and Urushigawa (19) reported that the biodegradation rate constants of alcohols by activated sludge increased as the carbon chain length of the alcohol increased from 1-butanol through 1-octanol. A later study by these researchers (16) with di-*n*-alkyl phthalate esters showed the reverse relationship. As the length of the alkyl group increased, the transformation rate decreased.

The plots of log K_{ow} versus log k_b for environmental populations for the esters of 2,4-D are shown in Fig. 1. About 94% of the variance in the 2,4-D ester data could be accounted for by the regression analysis; the k_b values of 2,4-D esters correlated well with the K_{ow} values. For the chloro-ring-substituted phenoxyacetic ethyl esters, however, only 47% of the data variance could be accounted for by regression of k_b on K_{ow} (Fig. 2). The lack of correlation for the ethyl ester series led us to consider another correlation, that of the pK_a of this series on k_b. One might expect that as the number of chlorines (electron-withdrawing substituent) on the benzene ring increased, the ester linkage would be more labile to hydrolysis. A correlation coefficient of 0.29, however, was obtained, indicating that electronic effects were not governing the reaction.

If we modify the definition by Hansch and Fujita (4) of the rate-limiting conditions for biological responses to chemicals

TABLE 3. Water solubilities and K_{ow} values of esters of chlorinated phenoxyacetic acids

Compound	log water solubility (μM)	log K_{ow}
Methyl ester of 2,4-D (mp 38–39°C)	3.04	2.92
Ethyl ester of 2,4-D (bp 136°C, 192 Pa)	2.65	3.25
Propyl ester of 2,4-D (bp 147°C, 222 Pa)	1.75	3.62
Butyl ester of 2,4-D (bp 153°C, 22 Pa)	1.27	4.13
Hexyl ester of 2,4-D (bp 160°C, 74 Pa)	0.43	4.70
Octyl ester of 2,4-D (bp 170–172°C, 74 Pa)	−1.00	5.47
Ethyl ester of 4-chlorophenoxyacetic acid (mp 47°C)	3.58	2.59
Ethyl ester of 2,4,5-trichlorophenoxyacetic acid (mp 64°C)	1.15	3.86
Ethyl ester of 2,3,4,5-tetrachlorophenoxyacetic acid (mp 106–107°C)	0.59	4.20
Ethyl ester of pentachlorophenoxyacetic acid (mp 113°C)	−1.28	4.75

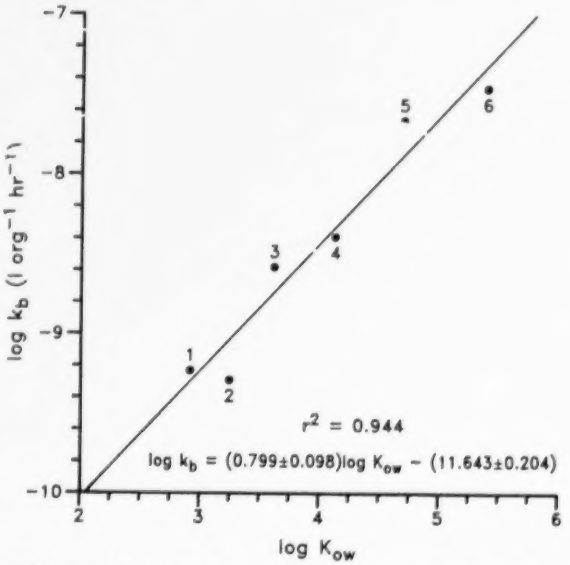

FIG. 1. Relationship between k_b values for environmental populations and K_{ow} values. Compounds are esters of 2,4-D: 1, methyl; 2, ethyl; 3, propyl; 4, butyl; 5, hexyl; and 6, octyl.

to fit bacterial systems, microbial responses can be defined as follows:

$$\text{compound S in water} \underset{}{\overset{k_b}{\rightleftharpoons}} \text{Enzyme (E)} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

Step 1 Step 2

$$\text{ES} \xrightarrow{k_2} \text{E} + \text{products}$$

Step 3

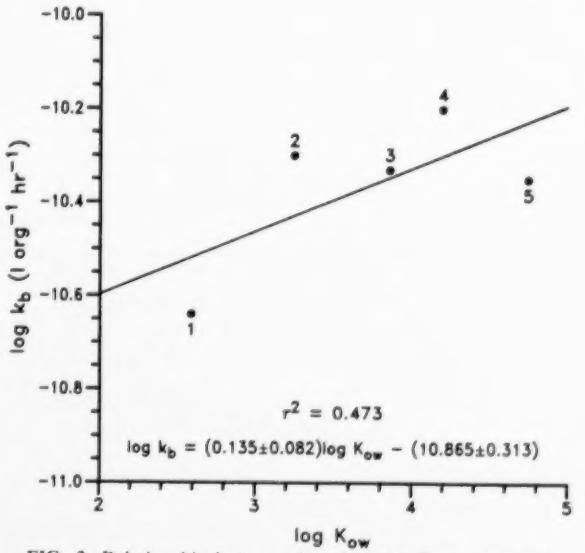

FIG. 2. Relationship between k_b values for *P. putida* and K_{ow} values. Compounds are ethyl esters of: 1, 4-chloro-; 2, 2,4-D; 3, 2,4,5-trichloro-; 4, 2,3,4,5-tetrachloro-; and 5, pentachlorophenoxyacetic acids.

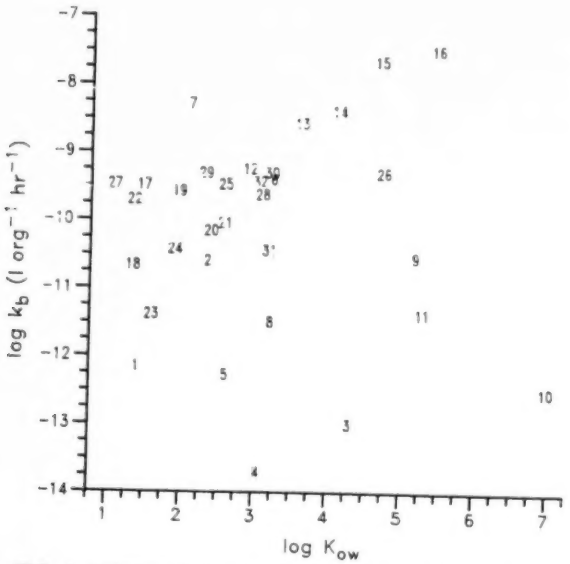

FIG. 3. Relationship between k_b values for environmental populations and K_{ow} values for 32 organic compounds: 1, methyl benzoate; 2, malathion; 3, methoxychlor; 4, chlorpropham; 5, methyl anisate; 6, butoxyethyl ester of 2,4-D; 7, dimethyl phthalate; 8, diethyl phthalate; 9, di-*n*-butyl phthalate; 10, di-*n*-octyl phthalate; 11, di-(2 ethyl hexyl) phthalate; 12 through 16, compounds 2 through 6 in the legend to Fig. 1; 17, phenol; 18, *p*-methoxyphenol; 19, *p*-cresol; 20, *p*-chlorophenol; 21, *p*-bromophenol; 22, *p*-acetylphenol; 23, *p*-cyanophenol; 24, *p*-nitrophenol; 25, ethyl ester of 4-chlorophenoxyacetic acid; 26, ethyl ester of pentachlorophenoxyacetic acid; 27, quinoline; 28, methyl parathion; 29, 9*H* carbazole; 30 dibenzothiophene; 31, benzo[*f*]quinoline; 32, compound 1 in the legend to Fig. 1. The k_b values were obtained from the following sources: 1 through 11 from reference 18; 12 through 16, 25, 26, and 32 from this paper; 17 through 24 from reference 11; 27 through 31 from reference 14. The K_{ow} values were obtained from the following sources: 2 and 4 from reference 7; 7 through 11 were calculated from K_{oc} (17) according to Karickhoff et al. (6); 12 through 16, 25, 26, and 32 were from this paper; 27 through 29 were calculated from K_{oc} (14) according to Karickhoff et al. (6); 1, 3, 5, and 6 were calculated from solubility according to Karickhoff et al. (6); 17 through 24 were from reference 10; 30 and 31 were from reference 14.

The equilibrium constant, K_b (15), was for sorption of the compound to the cell. For the compounds in this study, the lipophilicity of the molecule would be expected to govern steps 1 and 2—uptake of the compound across the cell wall or cell membrane and fit of the molecule to the lipophilic portion of the enzyme. The electronic properties of these molecules would be anticipated to be important in step 3.

It has been suggested that the rate of movement of numerous organic compounds across the cell wall or membrane is proportional to the log K_{ow} of the compound. For the 2,4-D *n*-alkyl esters in our study, K_{ow} and k_b increased together, but the relationship was inconsistent for the substituted ethyl series of esters. One possible reason for the lower dependence on log K_{ow} for these compounds could be that the site of action is located in the cell wall or membrane so that movement through lipophilic material is not necessary to reach the action site. Another reason could be that permeability of the cell wall or membrane (step 1) is not the rate-limiting step. Miller et al. (8), in studies of the antimicrobial activity of drugs, found that the rate-determining step in whole-cell systems and cell-free systems was similar, thus ruling out permeability as the rate-limiting step. If step 1 or movement of compounds to the site of action within the cell was the rate-limiting step in our systems, then k_b would be proportional to K_{ow} for all 10 esters. The fit of the ester to the enzyme in step 2 or to an active enzyme site in the cell wall or membrane appeared to be the limiting step for the 10 esters studied.

These studies suggest that attempts to correlate transformation of organic compounds in environmental waters based only on K_{ow} values can be misleading when terms like biodegradable, moderately biodegradable, etc., are considered. The hypotheses that the rate of uptake is the rate-determining step for all compounds and that the lipophilic factors are the most important factors are not supported. Our studies suggest that not only is the lipophilicity of the compound important but also the proximity to the reaction site. The plot of log k_b versus log K_{ow} (Fig. 3) indeed shows that such generalizations would be difficult to use for predictions.

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